Cell, Vol. 93, 103–109, April 3, 1998, Copyright ©1998 by Cell Press

The Unusual Active Site of Gal6/Bleomycin Hydrolase Can Act as a Carboxypeptidase, Aminopeptidase, and Peptide Ligase

Wenjin Zheng,* Stephen Albert Johnston,* and Leemor Joshua-Tor†‡
*Center for Biomedical Inventions
Departments of Medicine and Biochemistry
University of Texas-Southwestern Medical Center
Dallas, Texas 75235-8573
†W. M. Keck Structural Biology Laboratory
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

Summary

The Gal6 protease is in a class of cysteine peptidases identified by their ability to inactivate the anti-cancer drug bleomycin. The protein forms a barrel structure with the active sites embedded in a channel as in the proteasome. In Gal6 the C termini lie in the active site clefts. We show that Gal6 acts as a carboxypeptidase on its C terminus to convert itself to an aminopeptidase and peptide ligase. The substrate specificity of the peptidase activity is determined by the position of the C terminus of Gal6 rather than the sequence of the substrate. We propose a model to explain these diverse activities and Gal6's singular ability to inactivate bleomycin.

Introduction

Bleomycin hydrolase is an aminopeptidase originally discovered by virtue of its ability to hydrolyze the anti-cancer drug bleomycin, thus restricting its clinical usefulness (Akiyama et al., 1981; Lazo and Humphreys, 1983). Homologs have been cloned from yeast (Kambouris et al., 1992; Enenkel and Wolf, 1993; Magdolen et al., 1993; Xu and Johnston, 1994), bacteria (Chapot et al., 1993), chicken (Adachi et al., 1997), and mammals (Takeda et al., 1996), including human (Brömme et al., 1996; Ferrando et al., 1996). Studies of the yeast form of bleomycin hydrolase, Gal6p, have revealed it to be an unusual enzyme. The protein negatively regulates the galactose metabolism system and, surprisingly, binds single-stranded DNA and RNA with high affinity (Xu and Johnston, 1994; Zheng et al., 1997). This nucleic acid binding activity coupled to the hydrolase activity plays a role in the detoxification of bleomycin (Zheng and Johnston, 1997).

The crystal structure of Gal6p has been solved (Joshua-Tor et al., 1995), revealing two striking features. First, it is a ring, or barrel-like, structure consisting of six monomers that form a channel with an opening of about 22 Å in diameter and a central cavity of about 45 Å. Second, the six peptidase active sites, one in each monomer, are embedded in the structure such that they are only accessible by substrates through the central channel. Gal6 and the 20S proteasome are founding members of an increasing family of self-compartmentalizing or sequestered intracellular proteases (for reviews, Larsen and Finley, 1997; Lupas et al., 1997).

Direct external access to the active site of Gal6p is blocked by the C terminus of the protein, which inserts into the active site cleft as depicted in Figure 1. The C terminus is completely conserved in all forms of bleomycin hydrolase. The core of the Gal6 monomer, including the active site, is superimposable on the prototypic cysteine protease, papain. Gal6p, however, is an aminopeptidase with little substrate sequence specificity (Kambouris et al., 1992; Enenkel and Wolf, 1993; Magdolen et al., 1993; Zheng et al., 1997), whereas papain is an endopeptidase with specific substrate preferences, primarily for large hydrophobic residues at the S2 subsite (Schechter and Berger, 1968) ("S" refers to the protease subsite and the "P" to the substrate residues with numbers increasing away from the scissile bond). In cell extracts, Gal6p can account for all the bleomycin hydrolase activity.

We suspected that the substrate specificity of Gal6p could largely be determined by its unusually positioned and highly conserved C terminus. To investigate this possibility, we conducted structural and biochemical studies of Gal6p C-terminal variants. We show that the structure and position of the Gal6p C terminus controls the peptidase activity of Gal6p, allowing carboxypeptidase, aminopeptidase, endopeptidase, and/or peptide ligase activities in different C-terminal variants. The C-terminal architecture may explain why Gal6p is the only enzyme reported to date with the ability to detoxify bleomycin. Based on this understanding of the activity of Gal6, it may be possible to design inhibitors and cleavage-resistant bleomycin derivatives for clinical use.

Results

The Carboxypeptidase Activity of Gal6p

We first investigated Gal6p's apparent carboxypeptidase activity. Though the theoretical translation of the *GAL6* cDNA sequence terminates in a lysine, the crystal structure and peptide sequencing of Gal6p show that Lys 454 is missing in the native protein (Joshua-Tor et al., 1995). To determine whether Gal6p possesses a carboxypeptidase activity that might be responsible for the missing lysine, we solved the structure at 2.05 Å of a Gal6p variant in which the active site cysteine was changed to an alanine (C73A), thus destroying the peptidase activity. We find that the C-terminal amino acid in this variant, in contrast to the wild-type protein, is lysine (Figure 2A). As evident from the structure (Figure 2A) and cartooned in Figure 2C, the lysine (colored red) now occupies the P1 position in the binding pocket (Figure 2A). This position is open in the wild-type form (Joshua-Tor et al., 1995). The C73A variant has no other significant variation in structure from the wild-type. The root-mean-square (rms) deviation between the two is 0.39 Å for main chain atoms and 0.95 Å for all atoms present in both structures.

‡To whom correspondence should be addressed.

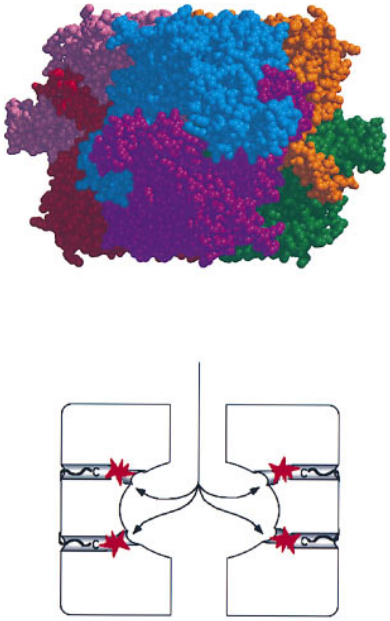

Figure 1. The Structure of the Gal6p Hexamer and Access to the Active Site

(Top) A space-filling representation of the molecule shown as a side view with the six subunits shown in different colors. This figure as well as Figures 2A and 2B were drawn with the program Molscript (Kraulis, 1991) and rendered with RASTER3D (Bacon and Anderson, 1988; Merritt and Murphy, 1994). The protein is shaped as a cylinder with a channel through its middle. The channel is approximately 20 Å in diameter at the two rims and opens to a larger central cavity. The C terminus of each subunit folds back into the active site cleft. (Bottom) A slice through the middle of the protein along it's 3-fold axis. Four out of the six active sites are shown as distorted stars and the C terminus as a squiggle line labeled at the end with the letter C. Access to the active sites is through the central channel.

To cleave Lys 454 from the C terminus, the Ala at position 453 would have to move to the P1 position and Lys 454 to the P1' position (Figure 2C). Such a conformation is exhibited in the structure of a variant with the active site Cys 73 changed to Ala and the C-terminal Lys 454 deleted genetically. This protein was purified and the crystal structure solved at 1.87 Å. In this case, as seen in the structure depicted in Figure 2B and cartooned in Figure 2C, Ala 453 moves to occupy the P1 position, displacing the Met 367 side chain, which is otherwise in the way. This moving forward of the C terminus is apparently allowed by the flexibility of the glycine at aa450. In the wild-type protein, this glycine forms a bulge in the C terminus (G bulge, yellow in Figure 2B). In the C73A/ΔK454 variant, this glycine rotates and extends by a screw-like movement, allowing Ala453 to move into the P1 position. Presumably, in the wild-type protein this would permit Lys 454 to occupy the P1' position for carboxypeptidase cleavage mediated by Cys73. In addition, in this variant one of the carboxylate oxygens of the terminal Ala453 occupies the "oxyanion hole" and interacts with glutamine 67 and the amide group of alanine 73 (formerly the catalytic cysteine). This configuration is postulated for the transition state in proteolysis and is similar to that found for the cysteine protease inhibitor, leupeptin (Schroder et al., 1993). The other carboxylate oxygen interacts with the catalytic

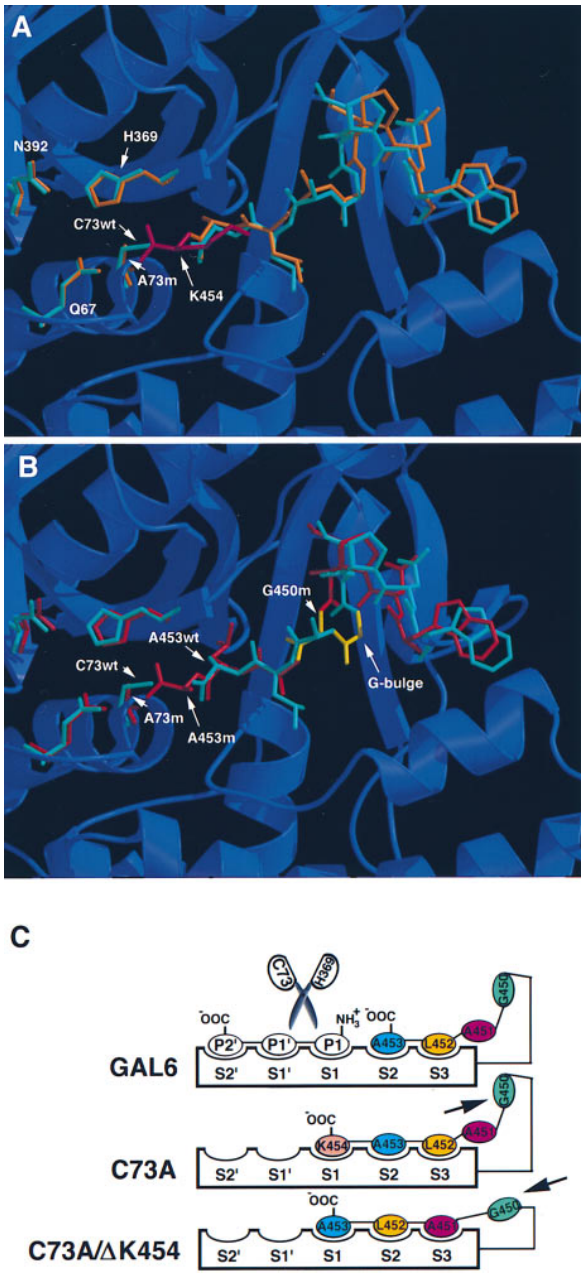

Figure 2. Positioning of the C-Terminal Arm of Gal6 in the Active Site Clefts in the Wild-Type and Mutant Proteins

(A) A superposition of the C-terminal arm and active site residues of the C73A variant (orange) on the active site cleft region of wild-type Gal6p (blue). The two C-terminal arms superimpose closely with K454 in the variant clearly seen in red as a well ordered residue occupying the S1 site and extending from the rest of the chain toward the catalytic residues.

(B) A similar superposition of the active site cleft region of C73A/ΔK454 (dark orange) superimposed on the wild-type protein (blue). A453 extends further toward the catalytic residues in this variant compared to wild type and is occupying the S1 site. The glycine bulge of the wild-type protein switches to an extended conformation in the variant (yellow).

(C) Cartoon illustrations of the active site structures of the wild type and the variants of Gal6p. Note that the C-terminal residues are color-coded in this and following cartoons. The scissors represent the active site cysteine and histidine at the cleavage site. In the Gal6 cartoon, the uncolored ovals represent the residues from the P1, P1', and P2' position of a peptide substrate.

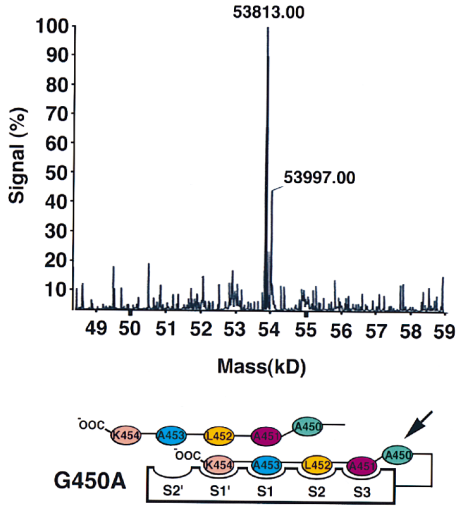

Figure 3. G450A Has a Different Processing Pattern from the Wild-Type Protein

An electrospray mass spectrometry shows G450A protein cleaving either one or three residues of the C terminus.

histidine (His 369). In the C73A variant the lysine carboxylate is in the same position. Since the oxyanion hole binds negatively charged oxygens, it is not surprising that one of the carboxylate oxygens in each of these variants would tend to bind there. This does not occur in the wild-type protein, possibly due to repulsion and/or steric hindrance by the sulfhydryl group of the active-site cysteine.

The G Bulge Is Involved in Accurate Autocatalytic Processing of K454

A prediction of the mechanism we propose for processing of the Gal6p C terminus is that the inherent torsion angle flexibility of Gly 450 plays a critical role in allowing correct positioning of the C terminus for carboxypeptidase activity. To test this prediction, we changed Gly450 to Ala genetically. We predicted that this alteration would modify the flexibility of the C terminus and therefore affect its normal processing. The G450A variant was purified from *Escherichia coli* and the molecular mass determined by electrospray mass spectrometry (Figure 3). A minority of the protein has the mass (53,997 Da) corresponding to Gal6p lacking the C-terminal lysine. However, the majority of the protein has the mass 53,813 Da corresponding to removal of the three C-terminal residues, Lys454, Ala453, and Leu452 (Figure 3). Therefore, Gly450 is crucial in obtaining the correct distance between the anchoring carboxylate and the nucleophilic cysteine. Interestingly, the protease cleaves between residues 451 and 452 but not between leucine 452 and alanine 453. Since the polypeptide chain in that region is in an extended conformation with the amide carbonyl groups alternating in opposite directions, only one direction is appropriate for nucleophilic attack by the cysteine sulfhydryl group, thus explaining why there is no detectable cleavage between residues 452 and 453.

We conclude that Gal6p processes its own C terminus by flexing it such that Lys454 is placed in the P1' position for cleavage. The glycine at 450 is crucial for this extension and precise cleavage. Once the lysine is removed, the C terminus retracts to place the now C-terminal alanine 453 at the S2 site. This processing in some ways resembles that of the Sindbid virus core protein, which also self-cleaves to leave its C terminus in the active site. However, in this latter case the processing is a single event, which yields an inactive enzyme (Tong et al., 1993).

The C Terminus of Gal6p Defines Its Substrate Specificity

The processing and the resulting positioning of the Gal6 C terminus implies that the C terminus itself is an important factor in determining the activity of the enzyme. Examination of the structure of the Gal6p active site suggests that the C terminus could serve two roles. The carboxylate of Ala453 could interact with the N terminus of a peptide substrate and anchor it in the active site. In addition, the C terminus could act as a "molecular ruler" to restrict the active site to aminopeptidase rather than endopeptidase activity.

To explore the role of the carboxylate, we altered the N terminus of the substrate. We found that Gal6p is active as an aminopeptidase on Lys-AMC (Lys-7-amido-4-methylcoumarin [Bachem], a fluorogenic substrate serving as a dipeptide analog) (K_{cat} = 19.3/s, K_m = 228 μM). However, when the acetylated form of this substrate, Ac-Lys-AMC, is used, there is a dramatic loss of activity (K_{cat} = 0.59/s, K_m = 243 μM). This neutralization of the N-terminal charge of the substrate leads to a 50-fold decline in K_{cat} with little or no effect on K_m. Similar results were obtained using other pairs of acetylated and nonacetylated substrates (data not shown). These results imply that the carboxylate interacts with the N terminus of the substrate and positions it by ionic interactions relative to the nucleophilic cysteine.

Converting Gal6p from Aminopeptidase to Endopeptidase

To investigate our proposed molecular ruler function of the C terminus, we made a series of Gal6p C-terminal deletions. Gal6Δ2 is a genetic deletion of Lys454 and Ala453, Gal6Δ3 deletes in addition Leu452, and Gal6Δ4 also deletes Ala451. Each of these proteins was purified from *E. coli* and is presumed to have an intact structure as each had normal DNA binding activity (data not shown). If indeed the substrate specificity of Gal6p is determined by the position of the C-terminal carboxylate, we predict that these variants would lose the ability to act on diamino-peptides and possibly become endopeptidases, since the anchoring carboxylate would position these short substrates too far from the nucleophile. As predicted, all three deletion variants have no detectable activity on the short Arg-AMC or Lys-AMC substrates. However, each of the deletions had activity on the longer Ala-Arg-AMC substrate ($K_{cat}/K_m\Delta2$ = 1/s/mM, $K_{cat}/K_m\Delta3$ = 3.2/s/mM, $K_{cat}/K_m\Delta4$ = 1.6/s/mM), albeit with a 40- to 100-fold lower K_{cat} than the wild-type protein on Arg-AMC ($K_{cat}\Delta2$ = 0.31/s, $K_{cat}\Delta3$ = 1.3/s, $K_{cat}\Delta4$ = 1.69/s).

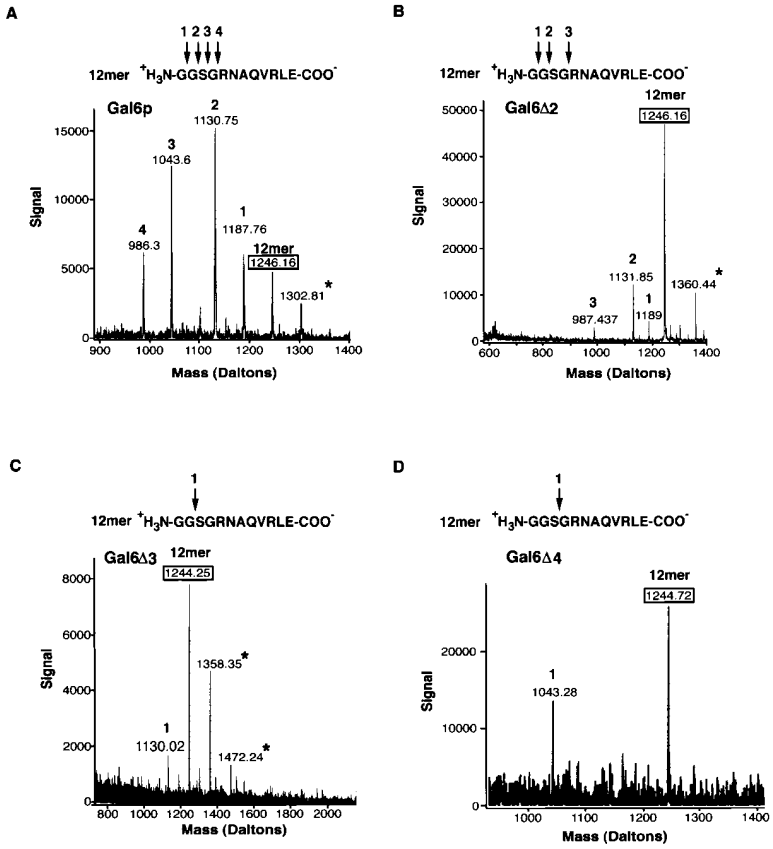

Figure 4. Mass Spectrometry Shows that Deletion of the C-Terminal Residues of Gal6p Changes Its Substrate Specificity

A 12 mer peptide was used as the substrate. The peptide sequence and the cleavage sites are indicated at the top of the picture (arrows). The mass of the 12 mer has been framed. (A) Gal6p. (B) Deletion of C-terminal two residues Gal6Δ2. (C) Deletion of C-terminal three residues Gal6Δ3. (D) Deletion of C-terminal four residues Gal6Δ4. In (A), (B), and (C), the peaks labeled by stars are ligation products resulting in an increase of mass over that of the original substrate.

We also tested the wild-type and variant proteins against a more natural substrate, a 12 amino acid residue peptide. The products were analyzed by mass spectrometry and showed that the wild-type protein created products that decreased by one amino acid sequentially (Figure 4A). This activity is a manifestation of aminopeptidase activity, as a time-course analysis of the products shows 11 mers evident first and shorter peptides accumulating at later time points (data not shown). In contrast to the wild-type protein, Gal6Δ2 (Figure 4B) and Gal6Δ3 (Figure 4C) cleave this substrate two amino acids at a time, and the Gal6Δ4 (Figure 4D) cleaves the 12 mer three amino acids at a time. That Gal6Δ2 and Gal6Δ3 cleave at one of the same positions and that Gal6Δ2 has a minor product resulting from a single amino acid cleavage probably arise from the restrictions on the hinge movement noted above. The results with both the artificial and natural substrates indicate that the deletion of C-terminal amino acids from Gal6p changes wild-type Gal6 from an aminopeptidase to an endopeptidase with cleavage of a particular peptide bond on the substrate depending on its distance from the C terminus of the enzyme rather than its surrounding sequence. Thus, the enzyme has positional specificity created by its particular placement of the C terminus.

The Peptide Ligase Activity of Gal6p and Its Variants

In the course of the studies using the 12 mer peptide substrate, we noted, surprisingly, that the deletion mutants produced products that were larger than the original substrate. For example, Gal6Δ3 produces products that are larger by two amino acid increments, in addition to the products that are two amino acids shorter, which are discussed above (Figure 4C). Indeed, on careful analysis the wild-type protein also produces a longer peptide in one amino acid increments (Figure 4A). Apparently, Gal6 and its C-terminal variants also have peptidyl ligase activity. This activity implies that in the initial acylation step the fragment N terminus to the scissile bond forms a relatively stable thioester intermediate with the enzyme. The C-terminal fragment must then be released so that new substrate can enter and be engaged in the transferase activity.

Increased Sensitivity of C-Terminal Deletion Mutant of Gal6p to Leupeptin

If the active site is left open by the C-terminal deletions, one prediction is that these endopeptidases would be sensitive to the inhibitor, leupeptin. As shown in Figure 5, the wild-type Gal6 protein is not sensitive to leupeptin, consistent with the observation that the C terminus occupies part of the position leupeptin would need to bind the active site (see Figure 4 in Joshua-Tor et al., 1995). In striking contrast, the Gal6Δ3 protein activity on the Ala-Arg-AMC substrate is sensitive to the inhibitor (Figure 5). These results provide a molecular explanation for the resistance of Gal6p to leupeptin; the processed carboxyl terminus of Gal6p protects the active site from inhibition.

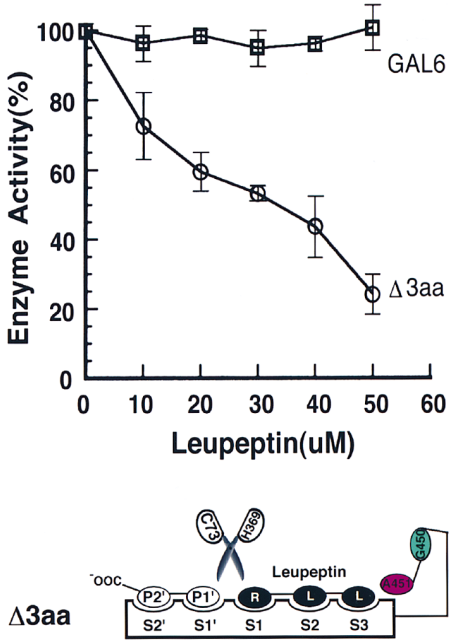

Figure 5. Deletion of the C Terminus of Gal6p Results in an Increase of Its Sensitivity to Leupeptin

(Top) Relative inhibition of wild-type and Gal6Δ3p by leupeptin on Arg-AMC and Ala-Arg-AMC substrates, respectively.

(Bottom) A cartoon illustrating the rationale for the increased sensitivity of deletions of the C terminus to leupeptin by opening subsites previously occupied by the C-terminal residues.

Discussion

In most enzymatic processes, substrate specificity occurs through clefts and crevices on the enzyme, which are lined with side chains or backbone groups that are complementary to the substrate. Based on the results of the experiments reported here, we present a different form of peptidase activity where substrate specificity is modulated by the positioning of Gal6p's own C terminus in the active site as opposed to the features of the substrate. It appears that the negatively charged C terminus of the enzyme anchors the positively charged amino terminus of a peptide substrate through ionic interactions. The site of cleavage is controlled by the distance of this C terminus from the nucleophilic active site residue as it acts as a molecular ruler to confer positional specificity.

One of the important implications of this model may be of clinical relevance, since it can be directly applied to bleomycin hydrolysis. Gal6p is apparently the only peptidase that can detoxify bleomycin in mammalian (Lazo and Chabner, 1996) and yeast (Xu and Johnston, 1994) cells. However, the C-terminal deletion variants of Gal6p do not detoxify bleomycin (Zheng and Johnston, unpublished data). Both of these observations may be explained by the positional specificity model. The amide bond of bleomycin that is hydrolyzed by Gal6p is on the β-aminoalanine moiety of the metal-binding region of the drug (Figure 6). We can consider the primary amino group of this moiety as the "amino terminus" of the

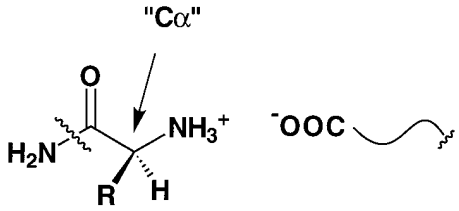

Figure 6. Possible Mechanism of Bleomycin Hydrolysis by Bleomycin Hydrolase

substrate, which can be anchored to the C-terminal carboxylate of Gal6; the carbon the amino group is bound to can then be considered to be the α-carbon of the first residue, with a chirality identical to an L-amino acid, and the amide bond that is hydrolyzed is the first peptide bond, which is cleaved in an aminopeptidase reaction. The rest of the bleomycin molecule could be considered as the "side chain." The chirality is such that this rather large side chain would point into the large cavity in the center of the Gal6 hexamer, just as would the side chain of the amino-terminal residue of any other peptide substrate. This view of how bleomycin is hydrolyzed may be useful in designing forms of the drug that are resistant to hydrolysis.

Another consequence of positional rather than sequence specificity is the ability to engineer variants with altered substrate specificity simply by changing the distance of the C terminus from the active site cysteine. Consistent with this observation, it was recently shown that deletion of the C-terminal residue of the bacterial bleomycin hydrolase, PepC, confers altered peptidase activity (Mata et al., 1997). In addition, two *GAL6* homologs were recently cloned from *Lactobacillus delbrueckii*, *pepG* and *pepW*, which have shorter C termini (WDSLA/I vs. WDPMGALA for yeast). Endopeptidase activity was demonstrated for *PepG* (Klein et al., 1997). These observations are consistent with the model presented here.

The organization and processing of Gal6p resembles that of the proteasome (Joshua-Tor et al., 1995; Löwe et al., 1995). Not only is the overall architecture similar with a channel leading to a large internal cavern, but, like Gal6p, the proteasome self-processes to activate its peptidase activity (Chen and Hochstrasser, 1996; Seemuller et al., 1996). For Gal6p, the proteosome, and other self-compartmentalizing proteases (Larsen and Finley, 1997; Lupas et al., 1997), it may be advantageous to sequester a nonspecific protease activity by spatial constraints while achieving some processivity by enclosing several catalytic centers within a single cavity. In addition, several of these self-compartmentalizing proteases do not belong to the classic families of aspartate, serine, cysteine, or metalloproteases, and those that do have unusual features as we have demonstrated for the C terminus in Gal6p and its involvement in substrate recognition. Clearly, there is much to discover about this structural class of proteases.

Experimental Procedures

Plasmid

The production of wild-type and C73A Gal6 proteins has been reported previously (Zheng et al., 1997). To construct C73A/ΔK454

mutant, the primer ActII-*GAL6* (5′-AAAAACCATGGCCTCTTCATCG ATA-3′) was used along with the primer *GAL6(-LYS)* (5′-AGAGCTGC TGAAACTATTTAGGCCAAAGCACCCATTGGGT-3′) to obtain the C73A/ΔK454 gene fragment by PCR using C73A gene fragment as a template. The resulting fragment was cloned into pKM260 as described previously (Zheng et al., 1997). The following oligonucleotides were used to delete the C-terminal residues of Gal6p. Three primers—*G6del2aa* (5′-CGGGATCCAGTTACAAAGCACCCATTGGG TCC-3′), *G6del3aa*, (5′-CTGAAACTATTTAAGCACCCATGGGGTC CCA-3′), and *G6del4aa* (5′-CGGGATCCAGTTAACCCATTGGGTCC CAGAT-3′)—were used with primer ActII-*GAL6* (5′-AAAAACCATGG CCTCTTCATCGATA-3′) to delete the C-terminal two, three, and four residues by PCR. The resulting fragments were digested by Nco1 and BamH1 and cloned into the BamH1/Nco1 site of pKM260 to form plasmids pWZ-BLHΔ2, pWZ-BLHΔ3, and pWZ-BLHΔ4. The plasmids were transformed into *E.coli* strain BL21 to express proteins. The G450A mutant was made by PCR *GAL6* gene fragment using the oligonucleotide MutGly (5′-CGCGGATCCCGCTATTTATTT GGCCAAAGCAGCCATTGGGTCC-3′) and primer ActII-*GAL6*. The resulting fragment was digested by BamH1/Nco1 and cloned into pKM260.

Protein Expression and Purification

For structure studies, mutant proteins were expressed in *E. coli* using a histidine tag followed by a TEV cleavage site at the N terminus. Purification to homogeneity was accomplished using Ni-NTA beads (Qiagen) followed by an anion exchange HQ perfusion chromatography column on a Sprint system (PerSeptives Biosystems).

The proteins for enzyme assays were prepared as follows: the *E. coli* strain BL21 was transformed with the appropriate plasmids for protein production. The resulting strains were grown in 2 ml cultures at 37°C overnight, each containing 25 μg/ml of ampicillin and chloramphenicol. Each culture then was inoculated into 1 liter of L-broth with the same antibiotics and grown to O.D. = 0.6–1.0. IPTG was added to a final concentration of 200 μg/ml. Cells were grown for another 12 hr at 16°C and then harvested. Proteins were purified using Ni-NTA resin following the standard protocol by Qiagen. Each protein from Ni-NTA beads was extensively dialyzed against Buffer A (25 mM Tris-HCl [pH 8.5], 10% glycerol, 50 mM KCl, 1 mM EDTA, 7 mM 2-Mercaptoethanol) and applied to a Bio-Scale Q5 column (BioRad) that had been equilibrated with Buffer A. The column was washed with 3–4 column volumes of Buffer A before the protein was eluted with a 0–0.5M KCl gradient in Buffer A. The peak fractions were pooled, dialyzed against Buffer A, and concentrated by Centricon 30 (Amicon). The protein was stored in this buffer plus 50% glycerol at −20°C.

Crystallization

Crystals of C73A were grown by the sitting drop vapor diffusion method. Equal amounts of a 20 mg/ml protein solution with 0.5 mM bleomycin in 25 mM Tris buffer (pH 8.5) were mixed with a reservoir solution containing 10% PEG 4K, 0.45 M $(NH_4)_2SO_4$ in 0.1 M Tris (pH 9.0). Crystals are of space group $P6_322$ with one monomer in the asymmetric unit. The cell dimensions are a = b = 150.68, c = 90.16. Crystals of the C73A/ΔK454 mutant protein were grown using the hanging drop vapor diffusion method. In this case, equal amounts of a 10 mg/ml protein solution with 0.5 mM bleomycin in 25 mM Tris buffer (pH 8.5) were mixed with a reservoir solution containing 0.5 M $(NH_4)_2SO_4$, 3% PEG monomethyl ether 2K in Tris (pH 8.5). The cell dimensions for these crystals are a = b = 150.55, c = 90.13 and are of the same space group.

Data Collection and Processing

A data set from a single crystal of C73A was collected at 90 K at beamline X12B at the National Synchrotron Light Source at Brookhaven National Laboratories using a 300 mm Mar Research imaging plate detector system and integrated and scaled with the HKL package (Otwinsowski, 1993). A total of 367565 reflections, 37272 unique with 97.3% completeness in the range 50.0–2.05 Å (99.2% in the last shell 2.12–2.05 Å) were collected. The R_{merge} for this data set is 7.7% (16.4% for the last shell).

For C73A/ΔK, a data set from a single crystal was collected at 90 K at beamline X25 at National Synchroton Light Source using a 300 mm Mar Research imaging plate detector system and integrated and scaled as for C73A. In this case, a total of 199,974 reflections were collected, 49,450 unique with 98.8% completeness at 35.0–1.87 Å resolution (96.5% in the last shell 1.94–1.87 Å). The R_{merge} for this data set is 4.2% (6.2% for the last shell).

Structure Determination

The structure of the C73A mutein was solved by isomorphous replacement using the wild-type protein structure (Protein Data Bank [PDB] code: 1GCB) with cysteine 73 changed to an alanine and excluding all solvent atoms. The initial R_{cryst} was 0.372 with an R-free (using 10% of the data) of 0.385. Rigid body refinement at 8–3 Å was followed by steps of positional refinement using X-PLOR (Brünger, 1988) with increasing resolution with iterative model building using the program O (Jones et al., 1991). Lysine 454 appeared very clearly in the electron density map and was added to the model. In addition, clear density appeared for five residues N-terminal to residue 1 belonging to the TEV cleavage site, which is part of the histidine tag used for this construct. These were added to the model as well. The final model was refined to 2.05 Å with a crystallographic R factor of 0.193 and free R of 0.257 with 3700 nonhydrogen protein atoms, 370 water molecules, and 6 sulfates. No σ cutoff was used. The rms deviation from ideality is 0.011 Å for bond lengths, 1.833° for bond angles, 21.6° for dihedral angles, and 1.44° for impropers. More than 90% of the (ϕ, ψ) angles of the model are in the most favored regions, and all residues are in allowed regions of the Ramachandran plot (Laskowski et al., 1993). The C-terminal region is very clear in the electron density maps. Coordinates were deposited in the PDB code 1a6r.

The structure of C73A/ΔK was solved and refined in much the same way as described for C73A. The structure was refined to 1.87 Å to a crystallographic R_{cryst} of 0.173 and free R of 0.217 with rms deviation from ideality of 0.012 Å for bond lengths, 1.722° for bond angles, 21.2° for dihedral angles, and 1.310° for impropers. The electron density is very clear in the region of the C terminus here as well. There are 420 water, 2 glycerol, and 5 sulfate molecules in the model. In this case more than 92% of the (ϕ, ψ) angles of the model are in the most favored of the Ramachandran plot and none in disallowed regions. Coordinates were deposited in the PDB code 3gcb.

Enzyme Assay

Gal6p protease activity was assayed with the synthetic substrate Arg-AMC (Bachem) under the condition as previously described (Xu and Johnston, 1994). Enzyme kinetic studies of Gal6p were accomplished on a luminescence spectrometer LS50-B (Perkin-Elmer). The excitation and emission wave lengths were 360 nm and 460 nm. The excitation and emission slit width were both 5 mm. Each assays has been repeated at least three times. The range of substrate concentration used was 0.1- to 4-fold of K_m. The rate of each reaction was determined by least square fit. The K_{cat} and k_m were obtained by Eadie-Scatchard plot.

The inhibition of leupeptin on Gal6p and the C-terminal three residue deletion mutein were assayed under the same condition as above except that 6 nM of the enzyme was used. For Gal6p, 100 μM of Arg-AMC was used, and for the mutant protein, 100 μM of Ala-Arg-AMC was used. Protein was incubated with different amount of leupeptin for 20 min at 4°C before being assayed at 30°C.

The peptide cleavages by Gal6p and the mutant proteins were assayed as follows: in a 20 μl reaction (50 mM KH_2PO_4, 50 mM Na_2HPO_4 [pH 7.5], 8 mM EDTA, 10 mM DTT), 2–10 μg of Gal6p (2 μg for Gal6p, 10 for deletion mutein) was mixed with 4 mM of 12 mer peptide. Reactions were incubated at 30°C for 1 hr (for Gal6p) or 2–4 hr for deletion mutant proteins. Two microliters of the reaction mixture was add to 200 μl of MALDI matrix buffer (50% acetone nitrile + 0.1% TFA) to stop the reaction. One microliter of which was used to measure the molecular mass by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) on Voyager DE (PerSeptive Biosystems).

Acknowledgments

We thank Julie Rosenbaum for protein purification and crystallizations, Dr. Malcolm Capel (at beamline X12B), Dr. Lonny Berman (at

beamline X25), and Dr. Felix Frolow for help and support with data collection at the National Synchrotron Light Source at Brookhaven National Laboratory. We thank Dr. David Corey, Dr. Clive Slaughter, and Dr. Meg Phillips (University of Texas-Southwestern) for help and lab facilities. We thank the Johnston and Joshua-Tor labs and Dr. Winship Herr for comments and suggestions. This work was supported by grants from National Institutes of Health (CA67982) and Tobacco Research Council (4247) to S. A. J., a Molecular Cardiology Training Fellowship of NIHLB to W. Z., and National Institutes of Health (R01-CA71746), the Arnold and Mabel Beckman Foundation, the Robertson Research Fund, and the Cold Spring Harbor Association Fellowship to L. J.

Received January 30, 1998; revised February 25, 1998.

References

Adachi, H., Tsujimoto, M., Fukasawa, M., Sato, Y., Arai, H., Inoue, K., and Nishimura, T. (1997). cDNA cloning and expression of chicken aminopeptidase H, possessing endopeptidase as well as aminopeptidase activity. Eur. J. Biochem. *245*, 283–288.

Akiyama, S., Ikezaki, K., Kuramochi, H., Takahashi, K., and Kuwano, M. (1981). Bleomycin-resistant cells contain increased bleomycin-hydrolase activities. Biochem. Biophys. Res. Commun. *101*, 55–60.

Bacon, D.J., and Anderson, W.F. (1988). A fast algorithm for rendering space-filling molecule pictures. J. Mol. Graph. *6*, 219.

Brömme, D., Rossi, A.B., Smeekens, S.P., Anderson, D.C., and Payan, D.G. (1996). Human bleomycin hydrolase: molecular cloning, sequencing, functional expression, and enzymatic characterization. Biochemistry *35*, 6706–14.

Brünger, A.T. (1988). Crystallographic refinement by simulated annealing: application to a 2.8 Å resolution structure of aspartate aminotransferase. J. Mol. Biol. *203*, 803.

Chapot, C.M., Nardi, M., Chopin, M.C., Chopin, A., and Gripon, J.C. (1993). Cloning and sequencing of pepC, a cysteine aminopeptidase gene from Lactococcus lactis subsp. cremoris AM2. Appl. Environ. Microbiol. *59*, 330–333.

Chen, P., and Hochstrasser, M. (1996). Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. Cell *86*, 961–972.

Enenkel, C., and Wolf, D.H. (1993). BLH1 codes for a yeast thiol aminopeptidase, the equivalent of mammalian bleomycin hydrolase. J. Biol. Chem. *268*, 7036–7043.

Ferrando, A.A., Velasco, G., Campo, E., and López-Otín, C. (1996). Cloning and expression analysis of human bleomycin hydrolase, a cysteine proteinase involved in chemotherapy resistance. Cancer Res. *56*, 1746–1750.

Jones, T.A., Zou, J.Y., Cowan, S.W., and Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Cryst. *A47*, 110–119.

Joshua-Tor, L., Xu, H.E., Johnston, S.A., and Rees, D.C. (1995). Crystal structure of a conserved protease that binds DNA: the bleomycin hydrolase, Gal6. Science *269*, 945–950.

Kambouris, N.G., Burke, D.J., and Creutz, C.E. (1992). Cloning and characterization of a cysteine proteinase from Saccharomyces cerevisiae. J. Biol. Chem. *267*, 21570–21576.

Klein, J.R., Schick, J., Henrich, B., and Plapp, R. (1997). Lactobacillus delbrueckii subsp. lactis DSM7290 pepG gene encodes a novel cysteine aminopeptidase. Microbiology *143*, 527–537.

Kraulis, P.J. (1991). Molscript: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. *24*, 946.

Larsen, C.N., and Finley, D. (1997). Protein translocation channels in the proteasome and other proteases. Cell, *91*, 431–434.

Laskowski, R.A., MacArthur, R.W., Moss, D.S., and Thornton, J.M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst. *26*, 283.

Lazo, J., and Chabner, B.A. (1996). In Cancer Chemotherapy and Biotherapy: Principles and Practice, 2nd edition, B.A. Chabner and D. Longo, eds. (Philadelphia: Lippincott-Raven Publishers).

Lazo, J.S., and Humphreys, C.J. (1983). Lack of metabolism as the biochemical basis of bleomycin-induced pulmonary toxicity. Proc. Natl. Acad. Sci. USA *80*, 3064–3068.

Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 Å resolution. Science *268*, 533–539.

Lupas, A., Flanagan, J.M., Tamura, T., and Baumeister, W. (1997). Self-compartmentalizing proteases. TIBS *22*, 399–404.

Magdolen, U., Muller, G., Magdolen, V., and Bandlow, W. (1993). A yeast gene (BLH1) encodes a polypeptide with high homology to vertebrate bleomycin hydrolase, a family member of thiol proteinases. Biochim. Biophys. Acta *1171*, 299–303.

Mata, L., Erra-Pujada, M., Gripon, J., and Mistou, M. (1997). Experimental evidence for the essential role of the C-terminal residue in the strict aminopeptidase activity of the thiol aminopeptidase PepC, a bacterial bleomycin hydrolase. Biochem. J. *328*, 343–347.

Merritt, E.A., and Murphy, M.E.P. (1994). Raster3D version 2.0: a program for photorealistic molecular graphics. Acta Cryst., *D50*, 869.

Otwinsowski, Z. (1993). In Data Collection and Processing, L. Sawyer, N. Isaacs, and S. Burley, eds. (Warrington, UK: Science and Engineering Research Council).

Schechter, I., and Berger, A. (1968). On the size of the active site in protease. 3. mapping the active site of papain; specific peptide inhibitors of papain. Biochem. Biophys. Res. Commun. *32*, 888–902.

Schroder, E., Phillips, C., Garman, E., Harlos, K., and Crawford, C. (1993). X-ray crystallographic structure of a papain-leupeptin complex. FEBS Lett. *315*, 38–42.

Seemuller, E., Lupas, A., and Baumeister, W. (1996). Autocatalytic processing of the 20S proteasome. Nature *382*, 468–471.

Takeda, A., Masuda, Y., Yamamoto, T., Hirabayashi, T., Nakamura, Y., and Nakaya, K. (1996). Cloning and analysis of cDNA encoding rat bleomycin hydrolase, a DNA-binding cysteine protease. J. Biochem. (Tokyo) *120*, 353–359.

Tong, L., Wengler, G., and Rossmann, M. (1993). Refined structure of Sindbis virus core protein and comparison with other chymotrypsin-like serine proteinase structures. J. Mol. Biol. *230*, 228–247.

Xu, H.E., and Johnston, S.A. (1994). Yeast bleomycin hydrolase is a DNA-binding cysteine protease: identification, purification, biochemical characterization. J. Biol. Chem. *269*, 21177–21183.

Zheng, W., and Johnston, S. (1997). The nucleic acid binding activity of bleomycin hydrolase is involved in bleomycin detoxification. Mol. Cell. Biol., in press.

Zheng, W., Xu, H., Eric, and Johnston, S.A. (1997). The cysteine-protease bleomycin hydrolase is a new component of the galactose regulon in yeast, J. Biol. Chem. *272*, 30350–30355.